RESEARCH

SP600125 suppresses *Keap1* expression and results in NRF2-mediated prevention of diabetic nephropathy

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Abstract

c-Jun N-terminal kinase (JNK) contributes to the pathogenesis of diabetic nephropathy (DN). The JNK inhibitor SP600125 was reported to ameliorate DN. However, the mechanism remained unclear. We previously reported that SP600125 activated nuclear factor erythroid 2-related factor 2 (NRF2), a governor of the cellular antioxidant defense system, in the aortas of the diabetic mice. Given the critical role of NRF2 in preventing DN, the present study aimed to test whether or not NRF2 is required for SP600125's protection against DN. To test the role of NRF2 in SP600125's effect, streptozotocin-induced C57BL/6 wild-type (WT) and *Nrf2*-knockout (KO) diabetic mice were treated in the presence or absence of SP600125, for 24 weeks. To explore the mechanism by which SP600125 activates NRF2, mouse mesangial cells (MMCs) were treated with high glucose (HG), in the presence or absence of either SP600125 or JNK siRNA. SP600125 significantly attenuated the diabetes-induced renal oxidative stress, inflammation, fibrosis, pathological change and dysfunction in the WT, but not the *Nrf2* KO mice. SP600125 inactivated JNK, inhibited kelch-like ECH-associated protein 1 expression, preserved NRF2 protein and facilitated its nuclear translocation in the kidneys of the WT mice, the effects of which were similarly produced by either SP600125 or JNK siRNA in HG-treated MMCs. Further, both SP600125 and JNK siRNA alleviated HG-induced mesangial oxidative stress and expression of inflammatory and fibrotic genes. The present study demonstrates that NRF2 is required for SP600125's protection against DN. SP600125 activates NRF2 possibly via inhibition of JNK-induced *Keap1* expression.

Key Words

- ▶ antioxidant
- ▶ diabetic nephropathy
- ▶ KEAP1
- ▶ JNK
- ▶ NRF2

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Introduction

Diabetic nephropathy (DN) is the main cause of end-stage renal disease (Dronavalli *et al.* 2008), and to date, has not been effectively treated. Therefore, it is essential to discover novel targets and develop more effective medicines to prevent the development of DN.

c-Jun N-terminal kinase (JNK) belongs to the mitogen-activated protein kinases (MAPK) family and regulates the transcription activity of activator protein 1 (AP-1) by phosphorylation of c-Jun (Davis 2000). AP-1 activates the transcription of a cohort of genes, such as transforming growth factor beta 1 (*Tgfb-1*) (Weigert *et al.* 2000) and fibronectin (*Fn*) (Lan *et al.* 2013), which play important roles in the initiation and progression of DN (Skena & Gesualdo 2005). Enhanced JNK activity was observed in high glucose (HG)-treated mesangial cells (Zhou *et al.* 2014, Gao *et al.* 2016), in kidneys of diabetic mice (Pan *et al.* 2013, Wang *et al.* 2015, Gao *et al.* 2016) and in kidney biopsy tissue from patients with DN (De Borst *et al.* 2007). Therefore, targeting JNK could be a promising strategy in prevention of DN. SP600125 is a specific inhibitor of JNK (Bogoyevitch *et al.* 2004). Previous studies reported the beneficial effect of SP600125 on DN (Wang *et al.* 2015, Gao *et al.* 2016, Hong *et al.* 2016). Despite the finding of decreased expression of several pro-fibrotic and pro-inflammatory genes, the mechanism through which SP600125 ameliorated DN was unclear.

Nuclear factor erythroid 2-related factor 2 (NRF2) is a master regulator of cellular antioxidant defensive activity (Zheng *et al.* 2011). As a transcription factor, NRF2 turns on the transcription of various antioxidant genes, such as heme oxygenase 1 (*Ho1*) and NAD(P)H dehydrogenase (quinone) 1 (*Nqo1*), leading to the increased antioxidants which function as scavengers for diabetes-induced free radicals (Ruiz *et al.* 2013). Accumulating evidence has demonstrated the pivotal role of NRF2 in self-defense against the pathogenesis of DN, since the *Nrf2* gene-knockout (KO) mice developed more severe nephropathy under diabetic condition, as compared with the wild-type (WT) mice (Jiang *et al.* 2010, Wu *et al.* 2015, 2016). In our previous study, SP600125 was found to activate NRF2 in the aortas of the diabetic mice (Liu *et al.* 2014), suggesting that JNK may negatively regulate NRF2. However, whether or to what extent NRF2 is required for SP600125's protective effect was unknown. By using *Nrf2* KO mice, the present study aimed to explore the role of NRF2 in SP600125's protection against DN.

Kelch-like ECH-associated protein 1 (KEAP1) is a key negative regulator of NRF2 (Kensler *et al.* 2007). KEAP1

sequesters NRF2 in cytoplasm, restricting NRF2 from nuclear translocation and promoting its proteasomal degradation (Zhang & Hannink 2003). JNK might be an upstream regulator of KEAP1, since 36 c-Jun-binding sites were found between -3000bp and -1bp within the promoter region of the mouse *Keap1* gene (Jaspar Database). Thus, JNK might inhibit NRF2 through induction of *Keap1* expression. By determining the renal level of *Nrf2* mRNA, the preservation of NRF2 protein, the proportion of the NRF2 nuclear translocation and the expression of *Keap1*, the present study tested the actions of SP600125 in the regulation of NRF2. To further verify the effect of JNK inhibition on NRF2 signaling and HG-induced injury, SP600125 was studied in HG-treated mouse mesangial cells (MMCs), by comparing to the effect of JNK siRNA.

In summary, the present study aimed to answer the following questions: (1) whether or not SP600125 activates NRF2 in DN; (2) whether or to what extent NRF2 is required for SP600125's protection against DN and (3) how does SP600125 activate NRF2.

Materials and methods

Animal treatment

C57BL/6 WT (*Nrf2*^{+/+}) and *Nrf2* KO (*Nrf2*^{-/-}) mice were obtained through breeding of heterozygotes (*Nrf2*^{+/-}) (Zheng *et al.* 2011, Dong *et al.* 2017). All mice were housed in the Animal Center of Jilin University at 22°C, on a 12:12-h light-darkness cycle with free access to rodent feed and tap water. The Institutional Animal Care and Use Committee at Jilin University approved all experimental procedures for these animals.

Eight-week-old male mice received either sodium citrate or streptozotocin (STZ, 50mg/kg daily, dissolved in 0.1M sodium citrate, pH 4.5; Sigma-Aldrich) through intraperitoneal injection for 5 consecutive days. One week after the last injection of STZ, fasting glucose levels (4-h fast) were measured. Mice with a fasting glucose level above 13.89mM were considered diabetic. Diabetic and age-matched control mice were then given by gavage with SP600125 (5mg/kg, dissolved in 1% CMC-Na solution; Medchem Express, Shanghai, PRC) or 1% CMC-Na solution, once every 2 days (Pan *et al.* 2014), for 24 weeks.

Blood glucose was recorded on days 0, 28, 56, 84, 112, 140 and 168 post diabetes onset. Urinary albumin and creatinine were recorded on day 168 post diabetes onset. The mice were then killed and their kidneys harvested for analysis.

Analysis of kidney dysfunction

A mouse albumin kit (Bethyl Laboratories, Montgomery, TX, USA) and a QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA) were used to determine urinary albumin and creatinine on spot urine samples following the manufacturer's instructions. Urinary albumin-to-creatinine ratio (UACR) was calculated to reflect kidney dysfunction.

Morphological analysis

After harvesting, the kidney tissues were fixed immediately in 10% buffered formalin solution and were embedded in paraffin and sectioned into 5- μ m-thick sections onto glass slides. The sections were processed for Periodic acid-Schiff (PAS) and Masson's trichrome staining. Morphometric analysis was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). Selection of areas to photograph and scoring was done by people blind to the identity of the samples.

Immunohistochemical (IHC) analysis

IHC staining was performed as previously described (Wu *et al.* 2014) to assess the status of renal oxidative damage and antioxidant activity, using primary antibodies against 3-nitrotyrosine (3-NT, Millipore; 1:100), HO1 (Santa Cruz Biotechnology, 1:100) and NQO1 (Santa Cruz Biotechnology, 1:100).

Cell culture and experiments

MMCs were isolated and cultured as previously described (Sun *et al.* 2017). Passages 5–7 were used for the experiments (Sun *et al.* 2017). To determine the effect of SP600125 and JNK siRNA on JNK activity, *Keap1* expression, NRF2 signaling and HG-induced injury, MMCs were subjected to HG (25 mM), in the presence or absence of 10 μ M SP600125 (Ho *et al.* 2007, Zhou *et al.* 2014, Gao *et al.* 2016) or JNK siRNA (100 nM, GenePharma, Suzhou, Jiangsu, PRC), for 48 h. The negative control siRNA was purchased from GenePharma. The transfection reagent Rfect was provided by Changzhou Bio-generating Biotechnologies, Changzhou, PRC.

Western blot analysis

Western blot analysis was performed using kidney cortex as previously described (Cai *et al.* 2005). The primary

antibodies were anti-connective tissue growth factor (CTGF, Santa Cruz Biotechnology, 1:500), anti-GAPDH (Santa Cruz Biotechnology, 1:3000), anti-histone H3 (Santa Cruz Biotechnology, 1:1000), anti-HO1 (Santa Cruz Biotechnology, 1:1000), anti-inducible nitric oxide synthase (iNOS, Cell Signaling Technology, 1:1000), anti-total cellular c-Jun (t-c-Jun, Cell Signaling Technology, 1:500), anti-total cellular JNK (t-JNK, Cell Signaling Technology, 1:1000), anti-KEAP1 (Santa Cruz Biotechnology, 1:500), anti-NQO1 (Santa Cruz Biotechnology, 1:1000), anti-NRF2 (Santa Cruz Biotechnology, 1:1000), anti-phosphorylated-c-Jun (p-c-Jun, Cell Signaling Technology, 1:500), anti-phosphorylated-JNK (p-JNK, Cell Signaling Technology, 1:500), anti-TGF- β 1 (Cell Signaling Technology, 1:1000), anti-tumor necrosis factor alpha (TNF- α , Cell Signaling Technology, 1:500) and anti-vascular cell adhesion molecule 1 (VCAM-1, Santa Cruz Biotechnology, 1:1000).

Real-time PCR

Quantitative real-time PCR was performed as previously described (Wang *et al.* 2009, Wu *et al.* 2014). Primers for collagen 4 (*Col4*), *Fn*, *Gapdh*, *Nqo1*, intercellular adhesion molecule 1 (*Icam-1*), *Ho1*, *iNos*, *Keap1*, *Nrf2* and *Vcam-1* were all purchased from Life Technologies.

Quantitative analysis of lipid peroxides

Renal malondialdehyde (MDA) concentration was calculated following the instructions by a lipid peroxidation assay kit from Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, PRC.

Isolation of nuclei

Nuclei of renal tissue and MMCs were isolated using a nuclei isolation kit (Sigma-Aldrich) as previously described (Dong *et al.* 2017, Sun *et al.* 2017), following the manufacturer's instructions.

Statistical analysis

Eight mice per group were studied. The measurements for each group were summarized as means $\pm$ s.d. Image Quant 5.2 (GE Healthcare Bio-Sciences) was used to analyze Western blots. Two-way ANOVA was performed for the comparisons among different groups of the two types of the mice. One-way ANOVA was performed for comparisons among different groups of either the WT or the *Nrf2* KO mice, as well as different groups of the cell

experiments. *Post hoc* pairwise comparisons were then performed using Tukey's test with Origin 8.6 data analysis and graphing software Lab (OriginLab, Northampton, MA, USA). A test is significant if $P < 0.05$.

Results

Deletion of the *Nrf2* gene led to a complete abolishment of SP600125's protection against diabetes-induced albuminuria and renal pathological change

Blood glucose levels were monitored every four weeks post diabetes onset. Both the WT and the *Nrf2* KO diabetic mice had significantly increased blood glucose levels at all the time points, as compared with their respective controls (Fig. 1A). SP600125 did not alter blood glucose under both the diabetic and the non-diabetic conditions, in both types of the mice (Fig. 1A). No significant difference in blood glucose level was detected between the WT and the *Nrf2* KO mice, under both the diabetic and non-diabetic conditions (Fig. 1A). However, as compared with the WT diabetic mice, the *Nrf2* KO diabetic mice developed remarkably higher UACR (Fig. 1B) and more severe renal pathological change, as shown by PAS staining (Fig. 1C) and Masson's trichrome staining (Fig. 1D), including the more drastic increase in glomerular area (Fig. 1E), mesangial matrix (Fig. 1F) and Masson's positive area (Fig. 1G). These results confirmed the critical role of NRF2 in self-defense against DN. Notably, in the WT mice, SP600125 significantly attenuated the diabetes-induced elevation of UACR (Fig. 1B, left panel) and pathological change (Fig. 1E, F and G, left panels). However, these beneficial effects of SP600125 did not exist in the *Nrf2* KO mice (Fig. 1B, E and F, right panels), with an exception of the amelioration of renal fibrosis (Fig. 1G, right panel). Nonetheless, the efficacy of SP600125 in decreasing renal fibrosis was blunted in the absence of NRF2, since SP600125 decreased renal fibrosis by 58.10% in the WT mice, whereas only by 25.47% in the *Nrf2* KO mice (Fig. 1G). Therefore, NRF2 plays a major role in SP600125's protection against the diabetes-induced albuminuria and renal pathological change.

NRF2 was required for SP600125's alleviation of the diabetes-induced renal oxidative stress, inflammation and fibrosis

Renal nitrosative damage was reflected by IHC staining of 3-NT (Fig. 2A), which showed a preferable expression

of 3-NT in the glomeruli. Further, renal oxidative stress, inflammation and fibrosis were evaluated at the molecular level. The levels of the oxidative stress indicators MDA (Fig. 2B) and iNOS protein (Fig. 2C), the protein levels of the pro-inflammatory markers TNF- α (Fig. 2D) and VCAM-1 (Fig. 2E), and the protein levels of the pro-fibrotic parameters TGF- β 1 (Fig. 2F) and CTGF (Fig. 2G) were measured in both types of the mice, all of which were elevated under the diabetic condition (Fig. 2B, C, D, E, F and G). SP600125 attenuated these indices in the WT, but not the *Nrf2* KO, diabetic mice (Fig. 2B, C, D, E and F), except for a mild decrease (26.74%) in CTGF in the *Nrf2* KO diabetic mice (Fig. 2G, right panel), in contrast to the WT mice (47.97%, Fig. 2G, left panel). These results indicate that NRF2 predominantly mediates SP600125's protection against diabetes-induced renal oxidative stress, inflammation and fibrosis.

SP600125 preserved renal NRF2 protein and facilitated NRF2 nuclear translocation and function, without altering *Nrf2* mRNA

Renal *Nrf2* expression and function were evaluated in the following study. Neither *Nrf2* mRNA (Fig. 3A, right panel) nor NRF2 protein (Fig. 3B, right panel) were detectable in the kidneys of *Nrf2* KO mice, as a confirmation of the *Nrf2* gene deletion. SP600125 did not alter *Nrf2* mRNA in the WT mice (Fig. 3A, left panel). Rather, it increased total cellular NRF2 (t-NRF2, Fig. 3B, left panel) and nuclear NRF2 (n-NRF2, Fig. 3C, left panel) in both the WT diabetic and non-diabetic mice. These results suggest that SP600125 regulates *Nrf2* expression at the protein, but not the mRNA level. NRF2 nuclear translocation was further evaluated by calculating the ratio of n-NRF2/histone H3 to t-NRF2/GAPDH, which was enhanced by SP600125 under both the diabetic and the non-diabetic conditions (Fig. 3D). Further, the mRNA levels of *Ho1* and *Nqo1* were both significantly increased by SP600125 in the WT, but not *Nrf2* KO mice (Fig. 3E and F). It is noted that, compared to the WT mice, the *Nrf2* KO mice expressed less renal *Ho1* and *Nqo1* mRNAs under both the diabetic and non-diabetic conditions (Fig. 3E and F), a result in line with the renal susceptibility of the *Nrf2* KO mice upon diabetes (Fig. 1B, C, D, E, F and G). Renal expression of HO1 and NQO1 was further assessed by IHC staining, which showed an increase of these antioxidants by SP600125 in the glomeruli of the WT mice (Fig. 3G and H, upper panels). This effect did not exist in the glomeruli of the *Nrf2* KO mice (Fig. 3G and H, lower panels). Collectively, SP600125 was found to activate NRF2 signaling via preserving NRF2

Figure 1

Deletion of the *Nrf2* gene led to a complete abolishment of SP600125's protection against diabetes-induced albuminuria and renal pathological change. Eight-week-old C57BL/6 WT and *Nrf2* KO male mice were induced to diabetes by streptozotocin. (A) Blood glucose levels were determined every four weeks post diabetes onset. Urinary albumin and creatinine were recorded at 24 weeks post diabetes onset with (B) UACR calculated. To evaluate renal pathological change, (C) PAS and (D) Masson's trichrome staining were performed, with (E) glomerular area and (F) mesangial matrix expansion quantified from PAS staining and (G) Masson's positive area quantified from Masson's trichrome staining. For (E, F and G), the data are normalized to WT Ctrl. All the data are presented as means $\pm$ s.d. ($n=8$). * $P<0.05$ vs WT Ctrl; $^{\dagger}P<0.05$ vs KO Ctrl; $^{\ddagger}P<0.05$ vs WT DM; $^{\S}P<0.05$ vs KO DM. Bar = 50 μ m. Scatter plots: solid up triangle, WT Ctrl; solid down triangle, WT Ctrl/JNKi; solid left triangle, WT DM; solid right triangle, WT DM/JNKi; hollow up triangle, KO Ctrl; hollow down triangle, KO Ctrl/JNKi; hollow left triangle, KO DM; hollow right triangle, KO DM/JNKi. Bars: white, Ctrl; light gray, Ctrl/JNKi; dark gray, DM; black, DM/JNKi. Ctrl, control; DM, diabetes mellitus; JNKi, the JNK inhibitor SP600125; KO, knockout; PAS, Periodic acid-Schiff; UACR, urinary albumin to creatinine ratio; WT, wild type.

Figure 2

NRF2 was required for SP600125's alleviation of the diabetes-induced renal oxidative stress, inflammation and fibrosis. To further test the role of NRF2 in SP600125's protection against diabetes-induced renal injury, IHC staining was performed to assess renal expression of (A) 3-NT, an indicator of nitrosative damage. Further, renal (B) MDA levels were measured by an MDA assay kit and protein levels of (C) iNOS, (D) TNF- α , (E) VCAM-1, (F) TGF- β 1 and (G) CTGF were determined by Western blot. For (B), the data is normalized to WT Ctrl. For (C, D, E, F and G), the data is normalized to either WT Ctrl or KO Ctrl, respectively. All the data are presented as means $\pm$ s.d. ($n=8$). * $P<0.05$ vs WT Ctrl; † $P<0.05$ vs KO Ctrl; ‡ $P<0.05$ vs WT DM; § $P<0.05$ vs KO DM. Bars: white, Ctrl; light gray, Ctrl/JNKi; dark gray, DM; black, DM/JNKi. 3-NT, 3-nitrotyrosine; CTGF, connective tissue growth factor; IHC, immunohistochemical; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; TGF- β 1, transforming growth factor beta 1; TNF- α , tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule 1. Other abbreviations are the same as those in Fig. 1.

protein and facilitating its nuclear translocation, rather than enhancing *Nrf2* transcription.

SP600125 decreased *Keap1* expression through inhibition of JNK activity

Given that KEAP1 restricts NRF2 from nuclear translocation and promotes proteasomal degradation of NRF2 (Zhang & Hannink 2003), we speculated that the observed preservation and relocation of NRF2 by SP600125 might be due to a downregulated KEAP1 expression or function. To test this hypothesis, renal *Keap1* expression was determined. As expected, SP600125 markedly decreased *Keap1* mRNA (Fig. 4A) and protein (Fig. 4B) in both types of the mice, under both the diabetic and the non-diabetic conditions. The inhibitory effect of SP600125 on JNK activity was further verified by the decreased phosphorylation of renal JNK (Fig. 4C) and c-Jun (Fig. 4D).

The following study was to further test whether inhibition of JNK activity could lead to a downregulated *Keap1* expression. Thus, HG-treated MMCs were subjected to either SP600125 or JNK siRNA. MMC was selected for the study because of the diabetes-induced remarkable pathological change observed in Fig. 1C, D, E, F and G. SP600125 decreased the ratio of p-JNK to t-JNK (Fig. 4E, left panel), but not t-JNK to GAPDH (Fig. 4E, middle panel). On the contrary, the ratio of t-JNK to GAPDH, but not p-JNK to t-JNK, was drastically decreased by JNK siRNA (Fig. 4E, left and middle panels). Despite these distinct actions of SP600125 and JNK siRNA (Fig. 4E, left and middle panels), both of them led to a significant decrease in the ratio of p-JNK to GAPDH (Fig. 4E, right panel). Inactivation of JNK by SP600125 and JNK siRNA was further confirmed by the decreased phosphorylation of c-Jun (Fig. 4F). SP600125 and JNK siRNA also led to an inhibition of *Keap1* expression at both the mRNA (Fig. 4G) and the protein (Fig. 4H) levels, the result of which was in accordance with the findings *in vivo* (Fig. 4A and B).

Taken together, by comparing with the effect of JNK siRNA, SP600125 was found to inhibit *Keap1* expression via inactivation of JNK.

Both SP600125 and JNK siRNA preserved NRF2 protein and enhanced its nuclear translocation and function

Given the verified JNK inactivation-induced inhibition of *Keap1* expression (Fig. 4G and H), the following study

Figure 3

SP600125 preserved renal NRF2 protein and facilitated NRF2 nuclear translocation and function, without altering *Nrf2* mRNA. To test the effect of SP600125 on *Nrf2* gene expression and function, (A) *Nrf2* mRNA, protein levels of (B) t-NRF2 and (C) n-NRF2 were determined in both types of the mice. In order to evaluate NRF2 nuclear translocation, (D) ratio of n-NRF2/Histone H3 to t-NRF2/GAPDH was calculated. Further, NRF2 function was evaluated by determining the mRNA expression of (E) *Ho1* and (F) *Nqo1*. IHC staining of (G) HO1 and (H) NQO1 was further performed to assess the status of renal antioxidant activity. The data are normalized to WT Ctrl and presented as means $\pm$ s.d. ($n=8$). * $P<0.05$ vs WT Ctrl; † $P<0.05$ vs WT DM. Bars: white, Ctrl; light gray, Ctrl/JNKi; dark gray, DM; black, DM/JNKi. *Ho1*, heme oxygenase 1; n-NRF2, nuclear NRF2; *Nqo1*, NAD(P)H dehydrogenase (quinone) 1; t-NRF2, total cellular NRF2. Other abbreviations are the same as those in Figs 1 and 2.

tested in HG-treated MMCs whether both SP600125 and JNK siRNA could preserve NRF2 protein and trigger its nuclear translocation. As shown in Fig. 5, both SP600125 and JNK siRNA increased t-NRF2 (Fig. 5A),

n-NRF2 (Fig. 5B) and ratio of n-NRF2/histone H3 to t-NRF2/GAPDH (Fig. 5C). The two JNK inhibiting approaches further enhanced the mRNA levels of *Ho1* and *Nqo1* (Fig. 5D and E).

Figure 4

SP600125 decreased *Keap1* expression through inhibition of JNK activity. With the aim of exploring the mechanism by which SP600125 activates NRF2, *Keap1* (A) mRNA and (B) protein, as well as ratios of (C) p-JNK to t-JNK and (D) p-c-Jun to t-c-Jun were determined in the kidneys of the WT and *Nrf2* KO mice. To further define the effect of JNK inhibition on *Keap1* expression, MMCs were treated with HG, in the presence of either SP600125 or JNK siRNA, with ratios of (E) p-JNK to t-JNK, t-JNK to GAPDH, p-JNK to GAPDH and (F) p-c-Jun to t-c-Jun, as well as *Keap1* (G) mRNA and (H) protein determined. For (A, B, C and D), the data is normalized to respective Ctrl and presented as means $\pm$ s.d. ($n=8$). * $P<0.05$ vs WT Ctrl; † $P<0.05$ vs KO Ctrl; ‡ $P<0.05$ vs WT DM; § $P<0.05$ vs KO DM. Bars: white, Ctrl; light gray, Ctrl/JNKi; dark gray, DM; black, DM/JNKi. For (E, F, G and H), the data are normalized to HG and presented as means $\pm$ s.d. ($n=3$). * $P<0.05$ vs HG; † $P<0.05$ vs HG/NC. Bars: white, HG; light gray, HG/JNKi; dark gray, HG/RFect^{PM}; black, HG/NC; white with stripes, HG/siJNK. HG, high glucose; *Keap1*, Kelch-like ECH-associated protein 1; NC, negative control siRNA; p-c-Jun, phosphorylated c-Jun; p-JNK, phosphorylated JNK; RFect^{PM}, the transfection reagent; siJNK, JNK siRNA; t-c-Jun, total c-Jun; t-JNK, total JNK. Other abbreviations are the same as those in Fig. 1.

Figure 5

Both SP600125 and JNK siRNA preserved NRF2 protein and enhanced its nuclear translocation and function. In order to test the effect of JNK inhibition on NRF2 expression and function, MMCs were subjected to HG, in the presence of either SP600125 or JNK siRNA. (A) t-NRF2 and (B) n-NRF2 levels were determined by Western blot. (C) n-NRF2/Histone H3 to t-NRF2/GAPDH was calculated to reflect NRF2 nuclear translocation. mRNA levels of (D) *Ho1* and (E) *Nqo1* were determined by RT-PCR. The data are normalized to HG and presented as means $\pm$ s.d. ($n=3$). * $P<0.05$ vs HG; † $P<0.05$ vs HG/NC. Bars: white, HG; light gray, HG/JNKi; dark gray, HG/RFect^{PM}; black, HG/NC; white with stripes, HG/siJNK; Abbreviations are the same as those in Figs 3 and 4.

JNK inhibition by either SP600125 or JNK siRNA decreased oxidative damage and expression of inflammatory and fibrotic genes in HG-treated MMCs

Finally, comparing to the effect of JNK siRNA, the role of JNK inhibition in the protective effect of SP600125 was tested by determining MDA levels (Fig. 6A) as well as mRNA expression of *iNos* (Fig. 6B), *Vcam-1* (Fig. 6C), *Icam-1* (Fig. 6D), *Fn* (Fig. 6E) and *Col4* (Fig. 6F) in HG-treated MMCs. All these parameters were significantly decreased by SP600125 or JNK siRNA (Fig. 6A, B, C, D, E and F).

Figure 6

JNK inhibition by either SP600125 or JNK siRNA decreased oxidative damage and expression of inflammatory and fibrotic genes in HG-treated MMCs. SP600125 and JNK siRNA were further tested for their roles in alleviating HG-induced oxidative stress, inflammation and fibrosis in MMCs. (A) MDA levels and mRNA expression of (B) *iNos*, (C) *Vcam-1*, (D) *Icam-1*, (E) *Fn* and (F) *Col4* were determined. The data are normalized to HG and presented as means $\pm$ s.d. ($n=3$). * $P<0.05$ vs HG; † $P<0.05$ vs HG/NC. Bars: white, HG; light gray, HG/JNKi; dark gray, HG/RFect^{PM}; black, HG/NC; white with stripes, HG/siJNK. *Col4*, collagen 4; *Fn*, fibronectin. Other abbreviations are the same as those in Figs 2 and 4.

Hence, the benefits of SP600125 were mediated by JNK inactivation.

Discussion

The present study researched the action of SP600125 in *Nrf2* expression and function in the protection against DN. By using *Nrf2* KO mice, NRF2 was found to be the major factor through which SP600125 ameliorated DN. In the kidneys of the WT mice, SP600125 inactivated JNK, inhibited *Keap1* expression, preserved cellular NRF2 protein level and facilitated NRF2 nuclear translocation. By comparing to the effect of JNK siRNA, SP600125 was found to inhibit *Keap1* expression, activate NRF2

signaling and attenuate HG-induced deleterious effects in HG-treated MMCs via inactivation of JNK. The present study indicates a negative regulatory effect of JNK on NRF2 in DN possibly through JNK-induced *Keap1* expression and may thus provide JNK inhibition as a feasible strategy for prevention of DN.

By phosphorylating c-Jun and thereby activating AP-1 (Davis 2000), JNK induces the transcription of *Tgf- β 1* (Weigert *et al.* 2000) and *Fn* (Lan *et al.* 2013), both of which play key roles in the pathogenesis of DN (Skena & Gesualdo 2005). Inhibition of JNK by SP600125 decreased *Tgf- β 1* (Gao *et al.* 2016) and *Fn* (Wang *et al.* 2015, Gao *et al.* 2016) and ameliorated DN. It was therefore speculated that JNK may contribute to the development of DN by activating *Tgf- β 1* and *Fn*. In the present study, NRF2 was found to be another downstream target of JNK. Different from the positive impact on *Tgf- β 1* and *Fn* gene transcription, JNK negatively regulated *Nrf2* expression at the protein level, but not the mRNA level (Fig. 3A and B). NRF2 played a major role in the protection by SP600125, since most of the beneficial effects of SP600125 did not exist in the absence of NRF2 (Figs 1B, C, D, E, F; 2A, B, C, D, E and 3E, F). Inhibition of *Tgf- β 1* and *Fn* by SP600125 might also play important roles in SP600125's protection against DN. However, this hypothesis needs to be further tested by manipulating *Tgf- β 1* and *Fn* expression in the presence of SP600125 in experimental models of DN. One possibility might be that NRF2, TGF- β 1 and FN all play pivotal roles, with crosstalk existing among them, functioning at different steps as a circuit or a cascade. Supporting this view, higher expression of *Tgf- β 1* and *Fn* was found in the kidneys of the *Nrf2* KO diabetic mice, as compared with the WT diabetic mice (Jiang *et al.* 2010, Wu *et al.* 2016). This indicates that NRF2 negatively regulates *Tgf- β 1* and *Fn* expression. Thus, upon diabetes, in addition to the JNK-induced AP-1 transcriptional activity, JNK might enhance *Tgf- β 1* and *Fn* expression via inhibition of NRF2. The correlation between NRF2, TGF- β 1 and FN in DN needs to be further researched in future studies. In addition to the potential NRF2/TGF- β 1/FN network, other mechanisms, such as anti-inflammation (Pan *et al.* 2013) and anti-renin-angiotensin system (Hong *et al.* 2016) may also contribute to SP600125's protection. Notably, NRF2 activation by SP600125 might also account for the anti-inflammatory function of SP600125, given that SP600125 completely lost the ability to attenuate the pro-inflammatory factors TNF- α and VCAM-1 (Fig. 2D and E) in the absence of NRF2. In the present study, a slight, but significant, decrease in Masson's positive area (Fig. 1G, right panel), and CTGF protein (Fig. 2G, right

panel) was detected in the kidneys of the SP600125-treated *Nrf2* KO diabetic mice. These observations suggest a NRF2-independent mechanism, which plays a partial role in SP600125's attenuation of diabetes-induced renal fibrosis, although the outcome of this mechanism was minor compared to the potent effect of NRF2 (Figs 1B, C, D, E, F and 2A, B, C, D, E).

Small molecules have been designed to disable KEAP1. Dimethyl fumarate, bardoxolone methyl (Wang *et al.* 2014) and sulforaphane (SFN) (Zhang & Hannink 2003) are the well-known small molecules that modify critical cysteines within KEAP1 protein, leading to the release of NRF2 (Takaya *et al.* 2012). Among these KEAP1 inhibitors, dimethyl fumarate is currently in use for clinical treatment of multiple sclerosis (Gold *et al.* 2012), demonstrating NRF2 as a viable drug target. Different from these KEAP1 inhibitors, SP600125 was found, in the present study, to decrease *Keap1* expression through inactivation of JNK, followed by NRF2 activation. The present study may suggest inhibition of *Keap1* expression as a strategy to activate NRF2, in addition to structural modification of the KEAP1 protein. In addition, the present study also suggests JNK gene silencing as an effective approach to activate NRF2 (Figs 4E, F, G, H and 5A, B, C, D, E). Both SP600125 and JNK siRNA decreased the amount of p-JNK (Fig. 4E, right panel), which reflected an inhibited JNK activity. However, SP600125 and JNK siRNA were found, in the present study, to inactivate JNK through different mechanisms. Specifically, SP600125 inactivated JNK by inhibiting its phosphorylation (Fig. 4E, left panel), whereas JNK siRNA via decreasing the amount of JNK (Fig. 4E, middle panel).

Controversies exist within establishing the effect of MAPKs on the regulation of NRF2 signaling. It was assumed that activation of MAPKs may phosphorylate NRF2 at specific serine and threonine residues, thereby facilitating the release of NRF2 from KEAP1 (Lee & Surh 2005). However, opposed JNK activity and NRF2 expression were observed in experimental models of various diseases (Tan *et al.* 2013, Yenki *et al.* 2013, Sahu *et al.* 2015, Ye *et al.* 2015, Du *et al.* 2016). Additionally, our previous report showed that inhibition of JNK by SP600125 or C66 led to NRF2 activation in the aortas of the diabetic mice (Liu *et al.* 2014). In line with these studies, the present study demonstrated a negative impact of JNK on NRF2 expression (Figs 3B, C, D, E, F, G and 5A, B, C, D, E). Collectively, these findings shed light on diverse regulatory effects of JNK on NRF2. One possibility for the differential behaviors of JNK could be the difference between disease conditions or cell types. A specific effect of JNK may play

a major role under a certain condition. The final outcome may be the result of a combination of all the actions of JNK. In the present study, enhancing *Keap1* expression was the most predominant action of JNK in inhibiting NRF2. Although JNK-induced phosphorylation of NRF2 and adaptive response to the oxidative stress might play positive roles in activating NRF2, these possible effects could be minor, as compared with the effect of JNK on inducing *Keap1* expression.

In our previous study, SP600125 increased both *Nrf2* mRNA and protein in the aorta (Liu *et al.* 2014). However, renal *Nrf2* mRNA was not altered by SP600125 in the present study (Fig. 3A, left panel). This could be due to the variation of the JNK function in different organs or cell types. Nonetheless, our previous study did show an increased nuclear positive staining of NRF2 in the aorta (Liu *et al.* 2014), suggesting an enhanced NRF2 nuclear translocation by SP600125. SP600125's negative effect on *Keap1* expression has been further tested and verified in the present study (Fig. 4A, B, G and H). In our previous report, metallothionein (MT), a potent antioxidant, was shown to be an important downstream target of NRF2 in DN (Wu *et al.* 2015), since deletion of the *Mt* gene partially, but significantly, abolished the protective effects of the NRF2 activator SFN. The rest of the protection produced by SFN in the absence of MT might be due to the NRF2-induced activation of other canonical downstream targets, such as *Ho1* and *Nqo1*.

It is noted that different stages of diabetic complications may have different status of antioxidant defensive function. *Nrf2* expression and function were upregulated in the aorta (Liu *et al.* 2014), heart (Bai *et al.* 2013) and kidney (Cui *et al.* 2012) of mice 3 months post diabetes onset, which was a relatively early stage of diabetes in mice. The activation of NRF2 antioxidant system could be due to the compensatory response to the stimulation by diabetes-induced oxidative stress (Bai *et al.* 2013), the effect of which adaptively overcomes the diabetes-induced injury. However, after a long-term exposure to diabetes-induced oxidative damage, the NRF2 antioxidant system tends to lose this function. This is evidenced by the impaired *Nrf2* expression and function in the hearts and kidneys of diabetic mice, 6 months post the onset of diabetes (Bai *et al.* 2013, Wu *et al.* 2016). In fact, decreased *Nrf2* expression was also observed in the hearts of diabetic patients as a chronic complication of diabetes (Tan *et al.* 2011). This could possibly be caused by the impaired compensatory function of the antioxidant defensive response after a long-term exposure to hyperglycemia. In the present study, enhanced JNK

function and *Keap1* expression in the 6-month diabetic kidneys might account for the impaired NRF2 function. Further studies are needed to elucidate the mechanism for the impaired *Nrf2* expression and function at the late stage of diabetes. In the present study, although renal NRF2 signaling was decreased in the WT diabetic mice (Fig. 3B, C, D, E and F, left panels), the presence of the basal NRF2 did play a protective role in the prevention of DN, since the *Nrf2* KO diabetic mice developed more severe renal dysfunction (Fig. 1B) and pathological changes (Fig. 1C, D, E, F and G), as compared with the WT diabetic mice.

Taken together, the present study demonstrates, for the first time, that NRF2 plays a critical role in SP600126 prevention of DN. Other findings suggest JNK to be a negative regulator of NRF2 in DN, the effect of which may be mediated by JNK-induced *Keap1* expression. This study may provide a basis for understanding the effect of JNK in DN and may indicate JNK inactivation, JNK gene silencing and inhibition of *Keap1* expression as potential strategies for prevention of DN.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H W conceived the project. H W, J W and H Z designed the research. H Z, X L, S Z, Y J, Y L and Y S researched the data. H W, J W and H Z wrote the manuscript. J W, H W, Y J, H Z, X L, S Z, Y L and Y S stimulated discussion. H Z, X L, S Z, Y J, Y L, Y S, J W and H W reviewed and revised the manuscript. H W, J W and Y J provided funding. All the authors approved the version to be published.

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